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PRINCIPAL INVESTIGATOR: Eckhard Podack, Ph.D.

CONTRACTING ORGANIZATION: University of Miami School of Medicine
Miami, Florida 33101

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Miami, Florida 33101

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13. ABSTRACT *(Maximum 200 words)*

A fusion between the heat shock protein gp96 and the Fc portion of immunoglobulin IgG1 was created deleting simultaneously the endoplasmic reticulum retention signal of gp96. The product, gp96-Ig, was transfected into the lymphoma line EG7 to generate EG7-gp96-Ig. Transfected gp96-Ig is secreted by the tumor cells and can be detected by ELISA. While EG7 and mock transfected EG7 form tumors in mice, EG7-gp96-Ig is rejected. Rejection requires CD8 cells but is not dependent on CD4 cells. Rejection is unimpeded when monocyte/macrophages are inactivated by Carrageenan. Tumor-secreted gp96-Ig generates protective, tumor specific tumor immunity and CD8 dependent T cell memory. It is concluded that tumor secreted gp96-Ig results in specific activation of CD8 cells upon in vivo inoculation in mice. The molecular mechanism by which CD8 T cells are activated is under investigation.

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FOREWORD

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Table of Contents

Introduction:

Gp96 has been shown previously to chaperone peptides and, when isolated from tumor cells, to serve as specific immunogen for providing tumor specific immunity. In this proposal we plan to generate a secretory form of gp96, which normally is not secreted but retained in the endoplasmic reticulum. Secretion of gp96 may be achieved by deleting the endoplasmic reticulum retention signal and replacing it with the Fc portion of IgG. It was reasoned that secretory gp96-Ig, secreted by tumor cells could provide specific anti tumor immunity when used as a vaccine.

Body:

a. Tumor vaccination with secretory gp96-Ig

The work in the first year focused on the investigation of a lymphoma cell line transfected with gp96-Ig. This cell line forms tumors in C57Bl6 mice. The tumor expresses ovalbumin as surrogate antigen. A TCR transgenic mouse, OT1, is available expressing a T cell receptor on all CD8 T cells detecting the ovalbumin peptide SIINFEKL presented by Kb. The EG7 tumor system together with gp96-Ig transfected tumors EG7-gp96-Ig therefore affords all the components needed for an analysis of gp96-Ig as vaccine for tumor immunization.

Pursuing this model we have found that tumor secreted gp96-Ig is a powerful vaccine, specifically immunizing against the tumor by which it is secreted and eliciting a strong CD8 T cell mediated response.

These data are summarized in the appended manuscript which has been submitted for publication.

b. Generation of CTL responses in vitro using gp96-Ig transfected tumors

In these studies we made use of the TCR transgenic OT1 mice detecting SIINFEKL presented by Kb. We wished to determine whether secreted gp96-Ig could directly stimulate OT1 T cells or whether it required uptake and presentation via class I by dendritic cells. Three experiments are shown that give puzzling answers to this question. In Fig.1 OT 1 cells (CD8 Tg) were isolated by negative selection and mixed in microtiter plates with various stimulator cells as indicated. DCs are dendritic cells generated by GMCSF and IL4 culture of monocytes. EG7 are ovalbumin producing EL4 lymphoma cells (H2b) and EG7-gp96-Ig are the same cells transfected with gp96-Ig and secreting gp96-Ig with its chaperoned peptides. OT1 cells were incubated and activated for three days and during the final 16 hours H3-thymidine was added, to measure cell activation by the ensuing DNA synthesis. Dendritic cells dramatically increased the response of OT1 cells to EG7, presumably through cross priming by ova peptide. Whether DC engulfed EG7 or take up secreted ovalbumin is not clear from this experiment. Surprisingly EG7-gp96-Ig was less active in stimulating cross priming of OT 1 by DC. In contrast, EG7-gp96-Ig was considerably more active than EG7 in priming OT1 cells directly. Purified ova peptide (SIINFEKL) was unable to stimulate OT1 cells in the presence or absence of DC when added directly to the culture. Binding of ova-peptide to Kb on OT 1 cells will induce fratricide..

Similar results were obtained in Fig.2. In this experiment in addition we added 3T3-ova and 3T3-gp96-Ig- ova. The latter cell secretes gp96-Ig as verified by ELISA, some of which should chaperone

ovalbumin peptides, similar to gp96-Ig secreted by EG7. However, 3T3-ove and 3T3-ova-gp96-Ig had no stimulatory effect for OT1 in the presence or absence of DC. This finding would suggest that under the conditions used, DC are unable to take up ovalbumin or gp96-Ig ova and use it for cross priming of OT1 cells. The strong effect of EG7 plus DC on OT1 cells argues for a direct contribution of the lymphoma on DC or on OT1 together with DC.

The effect of EG7 and EG7-gp96-Ig on OT1 cells in the presence and absence of transfected 3T3 cells was further tested in Fig. 3. It was found that the addition of 3T3 to the OT1 cells caused inhibition of EG7 induced activation even when the 3T3 cells produced ovalbumin and gp96-Ig. Using spleen cells in this experiment and adding ova peptide shows the extent to which OT1 cells can be maximally activated.

We conclude from this series of studies that the activation of CD8 cells in this system is not described by cross priming through dendritic cells. It appears that direct antigen presentation of the lymphoma cell to OT1 cells is operative which is enhanced by the production of secreted gp96-Ig chaperoning -ova. This effect on CD8 cells is akin to a costimulatory effect and could suggest that CD8 cells are equipped with gp96-Ig receptors that deliver positive costimuli.

Key research accomplishments:

- Construction of gp96-Ig
- Transfection of various cells and measurement of gp96-ig secretion
- Delineation of in vivo immune response to tumors secreting gp96-Ig
- Demonstration of strict CD8 dependent immune response to secreted heat shock protein

Reportable outcomes:

Publications 98-99:

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- Du, Z., Ricordi, C., Inverardi, L., Podack, E. and Pastori, R. Efficient ex vivo inhibition of perforin and Fas-ligand expression by chimeric tRNA hammerhead ribozymes. Human Gene Therapy. 9:1551-1560, 1998.

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- Chiarle, R., Podda, A., Prolla, G., Podack, E., Thorbecke, G.; Inghirami, G. CD30 overexpression Enhances Negative Selection in the Thymus and Mediates Programmed Cell Death via a Bcl-2 Sensitive Pathway. 1999. J. Immunol.

- Podack, E. How to induce involuntary suicide: The need for dipeptidyl peptidase I. 1999. Proc. Natl. Acad. Sci. USA.

- Yamazaki, K., Spruill, G., Rhoderick, J., Spielman, J., Savaraj, N. and Podack, E. R. Shared tumor antigens presented by HLA A1 or A2 on small cell lung carcinoma (SCLC) detected by purified $CD8^+$ T cells. 1999. Cancer Res.

Patent:

- gp96-IG - UM97-14 patent filed.

Abstract

- Yamazaki, K., Spielman, J., Spruill, G., Podack, E. gp96 engineered for secretion of tumor peptides and for vaccination against Cancer. FASEB Experimental Biology 1998. San Francisco, California.

- Yamazaki, K., Spielman, J., Spruill, G., Podack, E. Induction of tumor immunity by gp96 secreted from engineered tumor cells. AACR Cancer Research March 1999.

Conclusions:

Tumor secreted gp96-Ig mediates a strong CD8 response that generates protective and limited therapeutic immunity. The molecular mechanism of CD8 T cell activation appears to rely primarily on

direct antigen presentation and gp96-Ig serving as a costimulus.

References:

Non applicable

Appendix:

- Yamazaki, K., Nguyen, T., Podack, E.R. Secreted heat shock protein gp96-Ig elicits CD8 cells for tumor rejection. 1999. Submitted to Journal of Immunology.
- Yamazaki, K., Spielman, J., Spruill, G., Podack, E. gp96 engineered for secretion of tumor peptides and for vaccination against Cancer. FASEB Experimental Biology 1998. San Francisco, California.
- Yamazaki, K., Spielman, J., Spruill, G., Podack, E. Induction of tumor immunity by gp96 secreted from engineered tumor cells. AACR Cancer Research March 1999.

Fig. 1

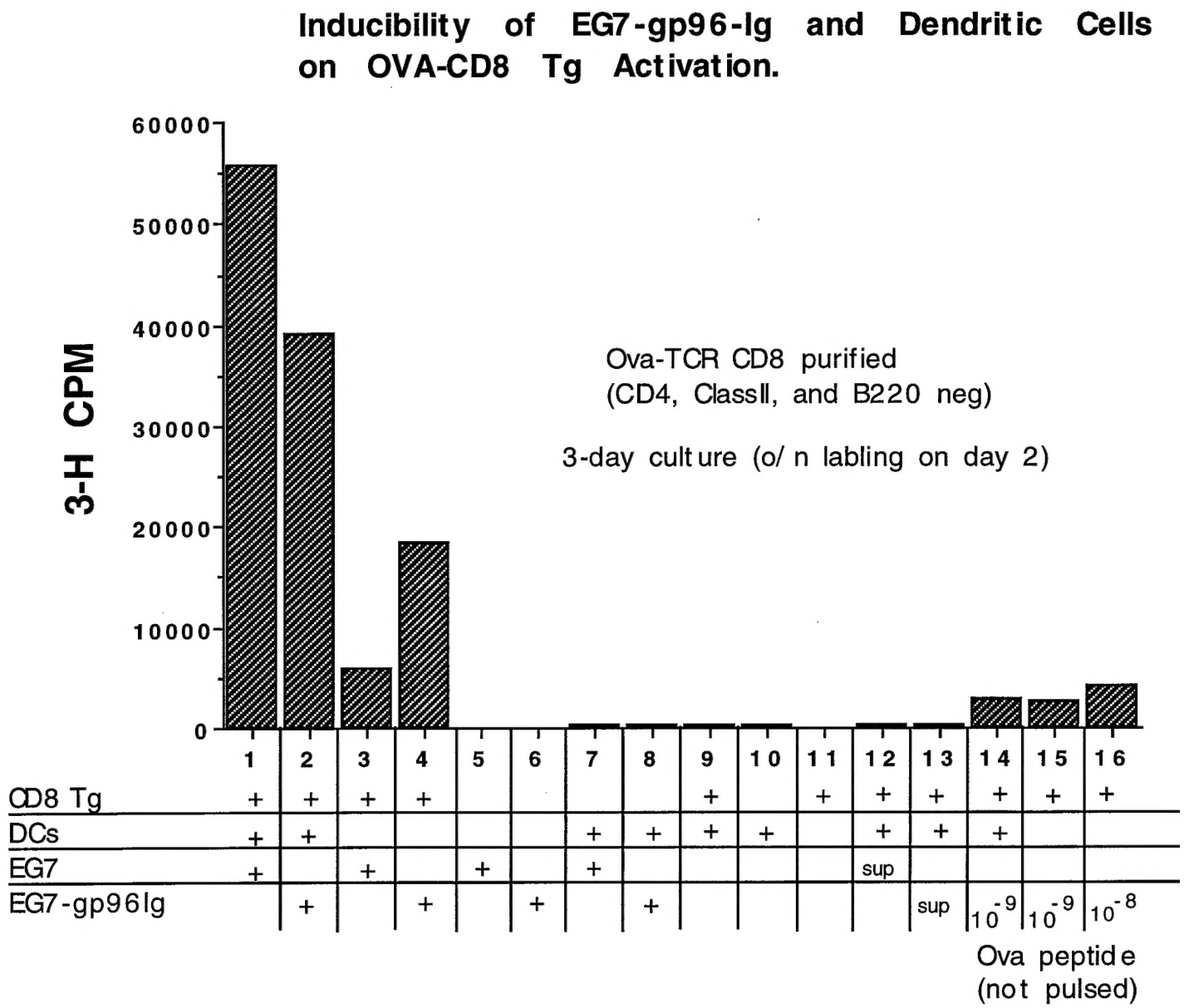

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Figure 2

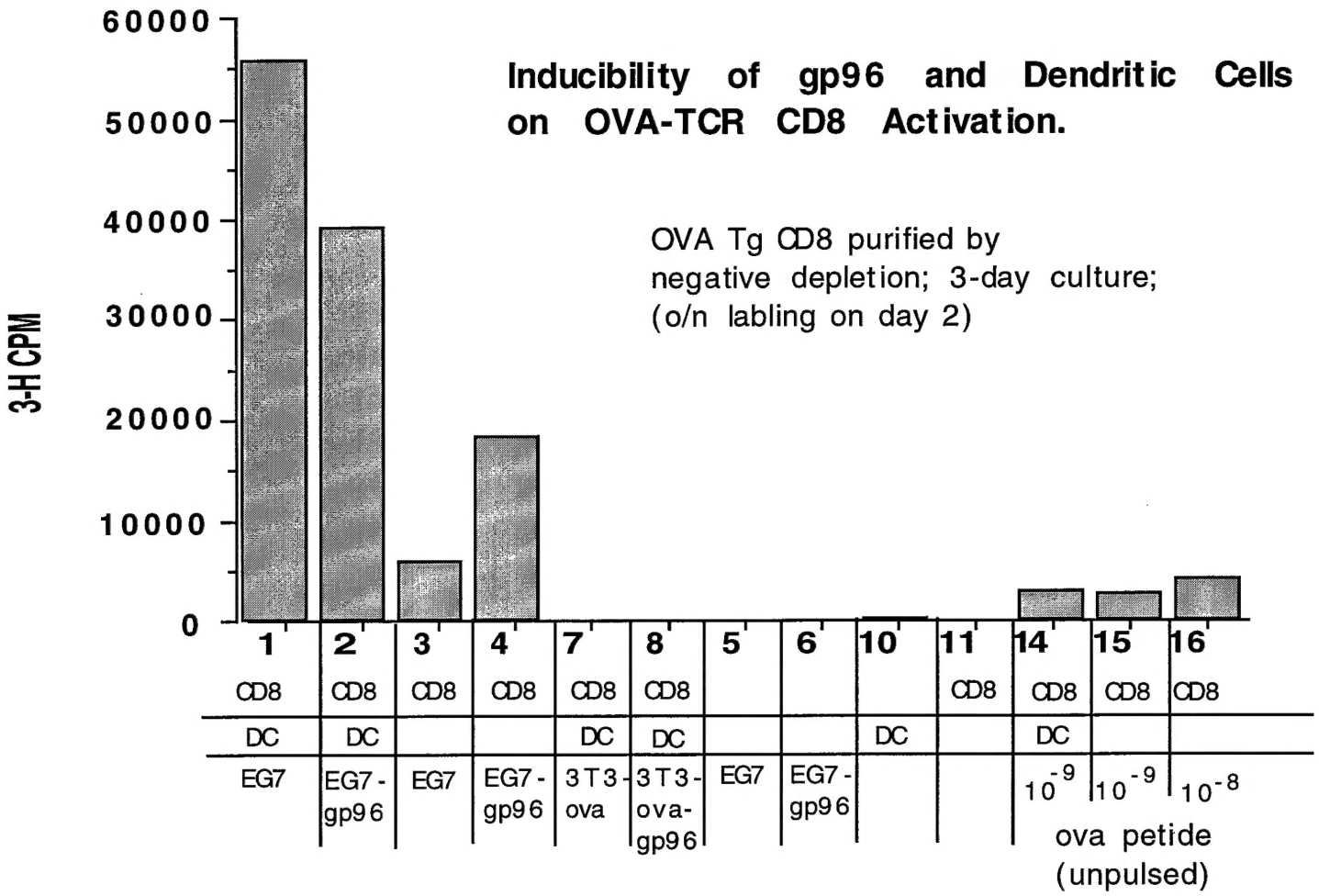

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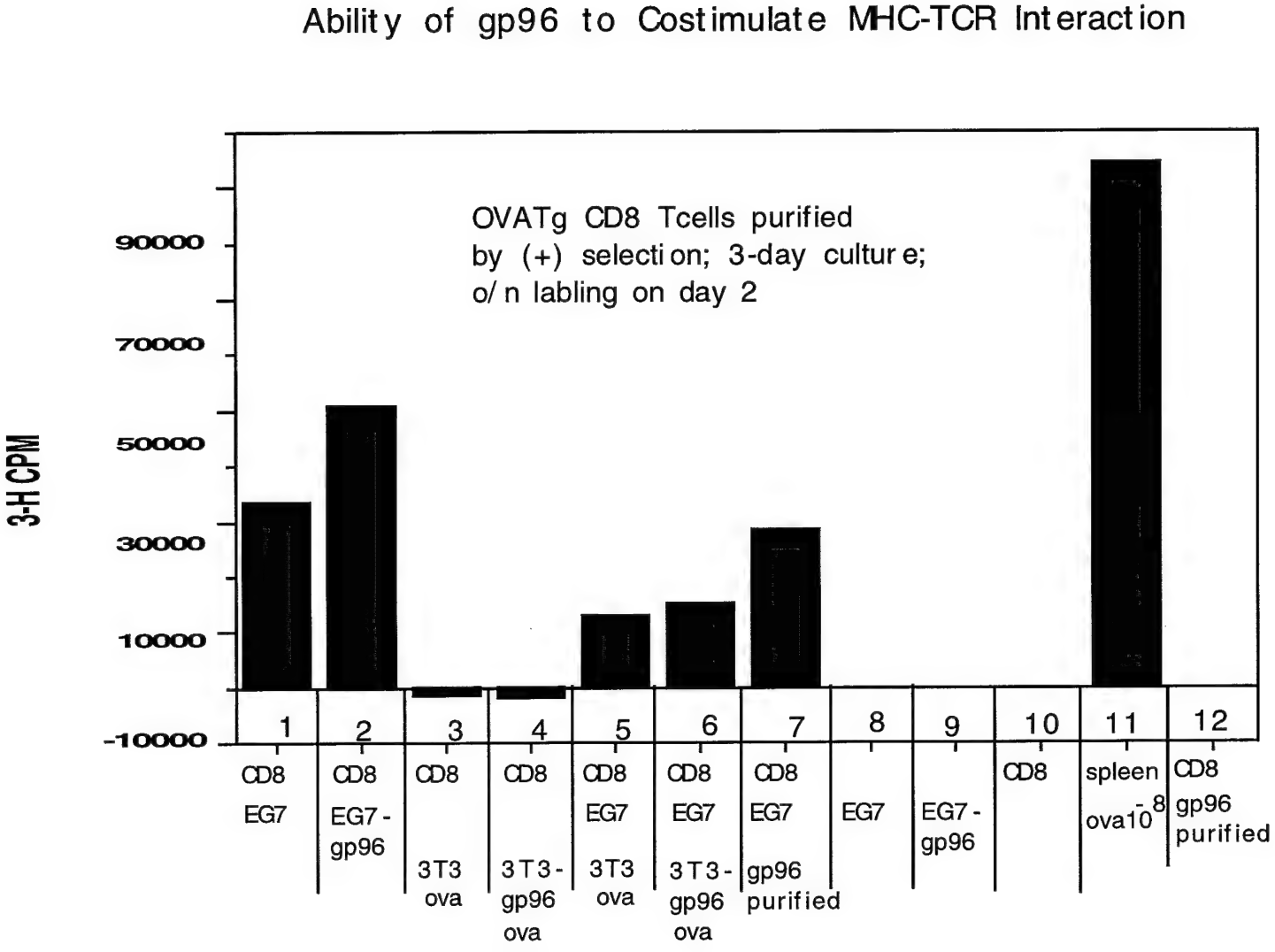
Figure 3



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Secreted heat shock protein gp96-Ig elicits CD8 cells for tumor rejection

Koichi Yamazaki, Timmy Nguyen and Eckhard R. Podack

Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, FL 33101

Correspondence should be addressed to

Eckhard R. Podack, M.D.

Professor

Department of Microbiology & Immunology

P.O. Box 016960 (R138)

Miami, Florida 33101

Phone: (305)243-6694

Fax: (305)243-5522

Email: epodack@miami.edu

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Abstract:

Purified heatshock protein gp96 isolated from tumor cells is highly immunogenic and induces tumor specific immune responses. The endoplasmic reticulum (ER)-resident gp96 appears to chaperon peptides, including those derived from tumor antigens, on their way to presentation by MHC class I. Replacement of the ER-retention signal of gp96 with the Fc portion of murine IgG1, in this study, generated a secretory form of gp96, gp96-Ig. Tumor cells transfected with gp96-Ig exhibited decreased tumorigenicity and increased immunogenicity in vivo and were rejected after initial growth. Rejection of tumors secreting gp96-Ig required CD8 T cells during the priming and during the effector phase while CD4 T cells were not required for rejection in either phase. Carrageenan, a compound known to inactivate macrophages in vivo, also did not diminish tumor rejection. Thus, immunization with tumors secreting gp96-Ig, unlike immunization with purified, tumor derived gp96, or with irradiated tumor cells, generates efficient tumor rejecting CD8 CTL without requirement for CD4 or macrophage help.

Introduction:

The heat shock protein (hsp) gp96, localized in the endoplasmic reticulum (ER), is thought to serve as a chaperon for peptides on their way to MHC class I and II molecules (1-4). Gp96-chaperoned peptides comprise the entire spectrum of peptides and larger protein fragments generated in cells and transported into the ER (5-9). Gp96 obtained from tumor cells induces specific tumor immunity (3, 10-13) presumably through the transport of tumor specific peptides to antigen presenting cells.

We developed a secretory form of gp96, gp96-Ig and tested it in tumor models. Transfection of tumor cells with the cDNA for gp96-Ig resulted in gp96-Ig secretion. As shown in this publication, gp96-Ig secreting tumor cells caused powerful immunization and tumor rejection in vivo.

Methods: *Cell lines.* All cell lines were obtained from the ATCC and cultured in medium with 10% FCS. Human small cell lung carcinoma cell lines (SCLC#2 and SCLC#7) were established as described (14).

Chicken ovalbumin cloned into the expression vector, pAc-Neo-Ova was kindly provided by Dr. M. Bevan (Seattle) (15) and used to transfect LLC. Transfected cells were selected with 1 mg/ml of G418 (GIBCO BRL) for at least 2 weeks and their secretion levels were tested by ELISA.

Antibodies were purchased from commercial sources.

Construction of gp96-Ig: In order to generate the gp96-Ig fusion protein, the KDEL sequence was deleted and replaced with the hinge, CH2 and CH3 domains of murine IgG1 (16-23); double-stranded cDNA was prepared from Jurkat DNA (24) with the GeneAmp RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT) and amplified by PCR using Pwo and Taq polymerase (Boehringer, Mannheim). The PCR primers were 5'-ATTACTCGA-GGGCCGCACGCCATGAGGG-3' and 5'-GCCCGGATCCTTCAGCTGTAGATTCCTT-TGC-3' (18, 19). The PCR primers included an XhoI site (forward primer) and a BamHI site (reverse primer). The hinge, CH2 and CH3 domains of murine IgG1 was amplified by using murine IgG1 cDNA as a template and mutating the three cysteines of the hinge portion to serines (21, 25). The PCR primers were 5'-GCGAGGATCCGTGCCCAGGGATTCTGGTTCTAAG-3' and 5'-CTAAGCGGCCGCAAGGACACTGGGATCATTTACCAGG-3'. The PCR primers included a BamHI site (forward primer) and NotI site (reverse primer). Gp96 was inserted into XhoI and BamHI sites of pBluescript, followed by insertion of murine IgG1 into the BamHI and NotI sites. Expression of the fusion protein, gp96-Ig, was confirmed by in vitro coupled transcription/translation (Promega, Madison, WI). Gp96-Ig cDNA was expressed with the eukaryotic expression vector, pBCMGSNeo and pBCMGHis (26-29) after transfection into SCLC#2, SCLC#7, B16F10, MC57,

LLC, NIH3T3, EL4, E.G7 and P815. Transfected cells were selected with 1 mg/ml of G418 or 2.5 -10 mM of L-Histidinol (Sigma, St.Louis, MO).

ELISA. Gp96-Ig secreting cells were plated at 10^6/ml in AIMV or IMDM with 10% FCS and culture supernatants were harvested at different time points. For analysis of intracellular expression of gp96-Ig, cells were lysed by three freeze-thaw cycles and centrifuged 60 min at 13,000 g (30). For ELISA flat-bottom, 96 well plates (Becton Dickinson Labware, Oxnard, CA) were coated with goat anti-mouse IgG (5 μg/ml) at 4°C overnight and blocked with 1% Gelatin in PBS at 37°C for 1 hr. Wells were incubated with culture supernatants or murine IgG (ICN, Costa Mesa, CA) as control at 37°C for 1 hr and developed with peroxidase-conjugated affinipure F(ab')2 fragment goat anti-mouse IgG (H+L) at 37°C for 1 hr, followed by incubation with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma).

Purification of gp96-Ig fusion protein. Gp96-Ig was purified by affinity chromatography on a Protein A column (Bio-Rad, Hercules, CA) (31). Gp96-Ig transfected NIH-3T3 cells were plated at 10^6/ml in AIMV and culture supernatants were harvested after 6-8 days. After removal of cellular debris by centrifugation and filtration, whole protein of the

supernatant was concentrated by ammonium sulfate precipitation (55% saturation) and dialyzed against PBS. Samples were diluted 1:2 with 3.5 M NaCl, 1.6 M Glycine, pH 9.0 (binding buffer) and applied to a Protein A column. The column was washed thoroughly with binding buffer, and bound protein was eluted with 0.1M citric acid, pH 6.5. Fractions containing protein were pooled and dialyzed against PBS. Concentration of gp96-Ig was determined by the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL).

SDS PAGE and Western blotting were done using standard procedures.

FACS analysis. For membrane staining of gp96-Ig, transfected SCLC cells were stained with goat anti-mouse IgG-FITC (to detect the murine Ig part of the fusion protein) or goat anti-rabbit IgG-FITC as a control for 15 min at 4°C and analyzed by a Becton Dickinson FACScan flow cytometer. For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with 1% saponin followed by staining with goat anti-mouse IgG-FITC, goat anti-mouse IgG-PE, goat anti-rabbit IgG-FITC or goat anti-syrian hamster IgG-FITC for 15 min at 4°C and analyzed by a flow cytometer.

Tumor inoculation and vaccination. Tumorigenicity in vivo was determined by subcutaneous injection of live tumor cells in 200 µl PBS

into the flanks of mice. The size of tumors was measured in two dimensions twice weekly for at least 2 months. When mean tumor growth exceeded 10 mm diameter, the mice were categorized as tumor positive and sacrificed. Mice were immunized by subcutaneous injection of 10^6 live E.G7-gp96-Ig or irradiated E.G7 as a control (in 200 µl PBS), given in the right flank. Two immunizations at 2 week intervals were given. Two weeks later, mice were challenged by subcutaneous injections of the indicated number of live tumor cells (EL4, E.G7, LLC or LLC-OVA in 200 µl PBS) into the left flank.

Depletion of T cells or macrophages in vivo. 100 µg of GK1.5 (anti CD4) or 2.43 (anti CD8) in 200 µl PBS was administered by intraperitoneal injection (32, 33). Depletion of CD4 and CD8 cells was verified by FACS analysis. For functional inhibition of macrophages, 1 mg of Carrageenan (type II; Sigma) in 200 µl PBS was administered by intraperitoneal injection (32, 34).

Results:

The endoplasmic reticulum-resident heatshock protein gp96 purified from tumor cells can provide tumor specific immunity (35). Gp96 carries tumor derived peptides that, upon injection, are taken up by host cells and are presented by class I MHC molecules to induce CTL responses. The

C-terminal sequence KDEL of gp96 serves as ER retention signal. Deletion of this sequence may be expected to result in the secretion of gp96 together with bound peptides from transfected tumor cells and may render tumors more immunogenic and allow tumor rejection by the immune system.

Characterization of secreted heatshock protein gp96

Replacing the KDEL sequence of gp96 with the hinge, CH2 and CH3 domain of murine IgG1 (Fig. 1a) and transfection of the cDNA into tumor cells resulted in the secretion of gp96-Ig into the culture supernatant, where it was quantitated by ELISA (Fig. 1b). Protein A purified gp96-Ig upon SDS page migrated with a major band of the predicted molecular weight of 120 kD for the fusion protein, and two minor, higher molecular bands, previously reported also for unmodified gp96 (Fig. 1c) (10). Western blotting with a monoclonal antibody specific for gp96 confirmed the identity of the fusion protein. Only the major band is stained suggesting that the minor bands are glycosylation variants of gp96 not recognized by the antibody.

Secretion of gp96-Ig resulted is time dependent with linear accumulation in the supernatant (Fig. 2). Intracellular gp96-Ig was detected at a low and constant steady state level in lysates of transfected

cells indicating that it does not accumulate in the cell. FACS analysis of membrane-intact, transfected tumor cells revealed no staining with anti mouse IgG above background demonstrating that the Ig moiety of the fusion protein is not displayed on the outer leaflet of the plasma membrane. In contrast, upon permeabilization of the membrane, gp96-Ig is detected intracellularly with a goat anti mouse IgG antibody, but not by control goat anti rabbit IgG antibodies. The transmembrane domain of gp96 does not interfere with the secretion of gp96-Ig and does not lead to intracellular accumulation. These data are consistent with previous reports suggesting that the transmembrane domain is not used for anchoring of gp96 in the membrane and that gp96 is not an integral membrane protein (36).

All murine and human cell lines transfected with gp96-Ig secreted the fusion protein (table 1). Mock transfected cells did not secrete gp96-Ig. Under standardized conditions (10^6 cells, 24h) the levels of secreted fusion protein varied depending on the transfectant from 5 ng/ml to 3300 ng/ml. Two cell lines were selected for further in vivo study. E.G7, an ovalbumin transfectant of the EL4 lymphoma, forms lethal tumors in syngeneic C57Bl/6 mice. Vaccination of C57Bl/6 mice with E.G7-gp96-Ig and testing for protection against EG7, EL4 and LLC-ova determines immunization against tumor associated antigens derived from ovalbumin

or the parent EL4 cell line. As second gp96-Ig transfected tumor for vaccination, the low-immunogenic, non-hematopoietic, LLC was used. Both cell lines secrete comparable amounts of gp96-Ig (table 1).

Secreted gp96-Ig mediates decreased tumorigenicity and increased immunogenicity

Secretion of gp96-Ig decreases the tumorigenicity of E.G7 in C57Bl/6 mice by more than a hundred fold when compared to mock transfected or untransfected E.G7. Subcutaneous inoculation of ten million heatshock protein secreting tumor cells caused tumors in only 10% of the inoculated mice (Fig. 3). A similar reduction of tumorigenicity by gp96-Ig secretion was observed with transfected EL4 (data not shown). Gp96-Ig secretion by LLC resulted in a more moderate, about five fold decrease of tumorigenicity (Fig. 3).

To determine immunogenicity and immune memory responses, C57Bl/6 mice were immunized twice at two week intervals with a dose of non irradiated, live E.G7-gp96-Ig (10^6) that was rejected. Subsequently they were challenged with untransfected or mock transfected E.G7, with parental EL4, with untransfected LLC and with LLC-ova (ovalbumin transfected LLC; Fig. 4). Mice immunized with irradiated E.G7 or unvaccinated mice served as controls. EG7-gp96-Ig immunized mice were

protected against a ten fold higher challenge dose of E.G7 than mice vaccinated with irradiated cells or unimmunized mice. The effect of immunization was even more pronounced when challenged with EL4, allowing a fifty fold dose increase of EL4 challenge compared to the controls. As expected, EG7-gp96-Ig immunization offered no protection against challenge with untransfected or vector transfected LLC while a moderate, about threefold increase in protection was observed when ovalbumin transfected LLC were used as challenge. The strong protection of mice immunized with EG7-gp96-Ig against EL4 challenge may be due to multiple tumor antigens shared by EG7 and EL4. The weak protection against challenge with LLC-ova, in contrast may be due to the limited number of epitopes derived from ovalbumin available for T cell recognition.

CD8 cells are required in the priming and effector phase

The involvement of immune mechanisms in the rejection of EG7-gp96-Ig was further examined by in vivo depletion/inactivation of immune competent cells. It has been reported that Meth A tumor derived gp96 requires CD4 and CD8 T cells for effective immunization in addition to macrophages while immunization with irradiated Meth A tumor cells required CD4 and CD8 cells but not macrophages (3).

To study the priming phase one million unirradiated, live EG7 secreting gp96-Ig were inoculated subcutaneously, a dose sufficient to establish tumors that grow to a mean diameter of about 8 mm diameter and subsequently shrink and are rejected. Tumor rejection is blocked in mice treated with the anti CD8 antibody 2.43, either two days prior (Fig. 5) to or up to three days after tumor inoculation (not shown). The anti CD4 antibody GK1.5 had no effect on tumor rejection (Fig. 5) regardless of time of injection, even though it completely depleted CD4 cells for more than 14 days (data not shown). Carrageenan, known to inactivate macrophages in vivo (34), had no effect on tumor rejection. CD4 deficient mice were able to reject EG7-gp96-Ig (Fig. 6) supporting the importance of CD8 cells.

To study the effector phase of tumor rejection, mice were immunized twice at 14 day intervals with live EG7-gp96-Ig. Eleven days later immune cell were depleted and after three days the mice were challenged with untransfected EG7 (Fig. 7). Only CD8 cells are required in the effector phase, depletion of CD4 cells or Carrageenan inactivation of macrophages had no influence on EG7 rejection in the effector phase.

Discussion:

Heatshock proteins transport peptides for binding to any MHC allele and therefore have the advantage of circumventing barriers to peptide

vaccination imposed by MHC restriction. Compared to DNA based vaccines heatshock vaccines avoid the risk of permanent genetic alteration. Heatshock proteins are intracellular proteins. Their isolation requires a substantial pool of cells and biochemical procedures.

Deletion of the endoplasmic retention signal of gp96 and replacement with the Fc portion if IgG1 results in the secretion of gp96-Ig. EG7 secreted gp96 is able to provide long specific immunity suggesting to chaperon tumor peptides. In contrast, irradiated or mock transfected EG7 are not able to provide protective immunity. Similarly, Corynebacterium parvum failed to serve as adjuvant for EG7 immunization (37). Secreted gp96-Ig provides immunologic specificity for both the surrogate antigen ovalbumin and other EL4 antigens, but does not cross immunize to LLC derived tumor antigens.

The data are consistent with the explanation that peptides associated with secreted gp96-Ig are transferred to and presented by class I MHC and stimulate tumor specific $CD8^+$ CTL causing tumor rejection. This response appears to be independent of CD4 help and does not require macrophages.

It may be instructive to compare the mechanisms of immunization by purified tumor derived gp96 and by live-tumor secreted gp96-Ig. Udono et

al. (32), using gp96 purified from Meth A tumor cells for immunization, reported a requirement for CD8 cells and macrophages in the priming phase and a requirement for CD4 and CD8 cells as well as macrophages in the effector phase of tumor rejection of Meth A tumors. Immunization with irradiated Meth A tumors required CD4 cells in the priming phase, and both CD4 and CD8 cells in the effector phase.

Irradiated EG7 do not produce immunity against subsequent challenge. The dramatic effect of EG7 secreted gp96-Ig is entirely dependent on CD8 cells without CD4 help. CD8 cells are required both in the priming and effector phase of the CTL response to the tumor. Macrophages are not needed. The role of dendritic cells or other antigen presenting cells in presentation of gp96 chaperoned peptides to CD8 cells is not known, but remains a possibility. It is also possible, that gp96-Ig secreting EG7 stimulate CD8 cells directly.

Regardless of the underlying mechanism, tumor secreted gp96-Ig is a powerful vaccine for some tumors. The precise mechanism CD8 CTL activation by tumor secreted gp96-Ig and its action in a diverse array of tumors remain to be determined.

Acknowledgements

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Figure legends:

Figure 1:

Construction, secretion and characterization of gp96-Ig. a: Schematic representation of the gp96-Ig construct. b. ELISA for gp96-Ig of supernatants from gp96-Ig cDNA transfected and untransfected small cell lung carcinoma line #7 (SCLC); cells were plated at 10^6/ml and supernatants tested on day 3 and day 6; purified mouse IgG (500 ng/ml) served as standard. c: SDS PAGE of protein A purified gp96-Ig. lane 1: Coomassie blue stain (1 μg protein), lane 2: Western blot with monoclonal anti gp96 (anti Grp94, 9G10) (100 ng protein)

Figure 2:

Localization of gp96-Ig. **a**: Accumulation of secreted gp96-Ig in the medium. Solid circles: gp96-Ig in the culture supernatant, open circles: gp96-Ig in cell lysates. GP96-Ig was quantitated by ELISA; SCLC-gp96-Ig was plated at 10^6/ml. **b**: Intracellular staining of gp96-Ig by FACS analysis of permeabilized SCLC-gp96-Ig; dashed line, goat anti rabbit IgG-FITC (negative control); solid line, goat anti mouse IgG-phycoerythrin. **c**: Absence of surface stain for gp96-Ig on unpermeabilized SCLC; left panel

untransfected, right panel gp96-Ig transfected SCLC. Dashed line in both panels is goat anti rabbit IgG-FITC; solid line, goat anti mouse IgG-FITC.

Figure 3:

Decreased tumorigenicity of gp96-Ig transfected E.G7 and LLC solid rectangles), in comparison to mock transfected (open circles) and untransfected cells (open rectangles). Groups of six mice were used per dose of inoculated cells.

Figure 4:

Secretory gp96-Ig vaccination generates tumor specific memory. C57Bl/6 mice were immunized twice in biweekly intervals with 10^6 gp96-Ig transfected E.G7 (solid squares in all panels), with 10^6 irradiated EG7 (open squares) or not immunized (open circles). Two weeks later mice were challenged (six mice per group) with the number of tumor cells as indicated in the panels.

Figure 5:

Effect of depletion of immuno-competent cells on the rejection of 10^6

E.G7-gp96-Ig during the priming phase; controls received PBS. Tumor growth curves in individual mice are shown. The depletion schedule is shown on top. Depletion of immune competent cells was done two days prior to tumor inoculation with 10^6 EG7-gp96-Ig.

Figure 6:

CD4 deficient mice can reject EG7-gp96-Ig. Five CD4 k/o mice were challenged with unirradiated 10^6 EG7-gp96-Ig subcutaneously. Tumor growth was recorded, and the mean tumor diameter is reported.

Figure 7: Effect of depletion of immune competent cells on the effector phase of EG7-gp96-Ig rejection. The schedule of immunization and immune depletion is shown on the top. For immunization 10^6 unirradiated EG7-gp96-Ig were inoculated subcutaneously into groups of six mice. Three days prior to rechallenge with 10^6 EG7, immune cells were depleted; controls received PBS. Tumor growth was recorded, and is reported as mean tumor diameter.

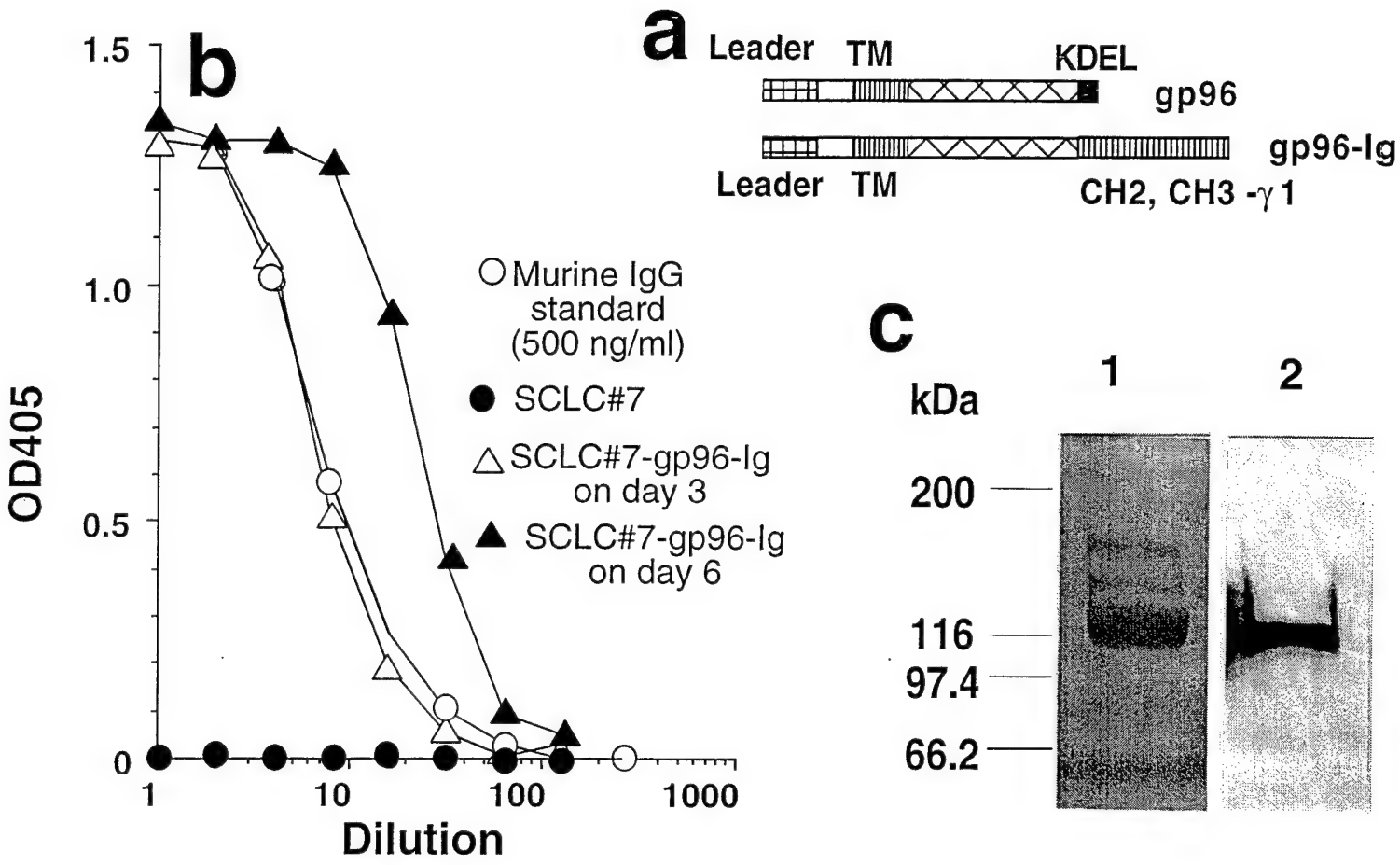
Table 1. Secretion of gp96-Ig into culture supernatants

Cell lines	Gp96-Ig/10^6cellsx24h
SCLC#2	140 ng
SCLC#7	500 ng
NIH3T3	500 ng
EL4	160 ng
E.G7	60 ng
P815	<5 ng
LLC	70 ng
B16F10	312.5 ng[a]
MC57	3,300 ng

[a] Metallothioneine promoter

Gp96-Ig cDNA was expressed in a bovine papilloma virus derived episomal vector under the CMV or metallothioneine promoter.

Fig. 1; Yamazaki et al.



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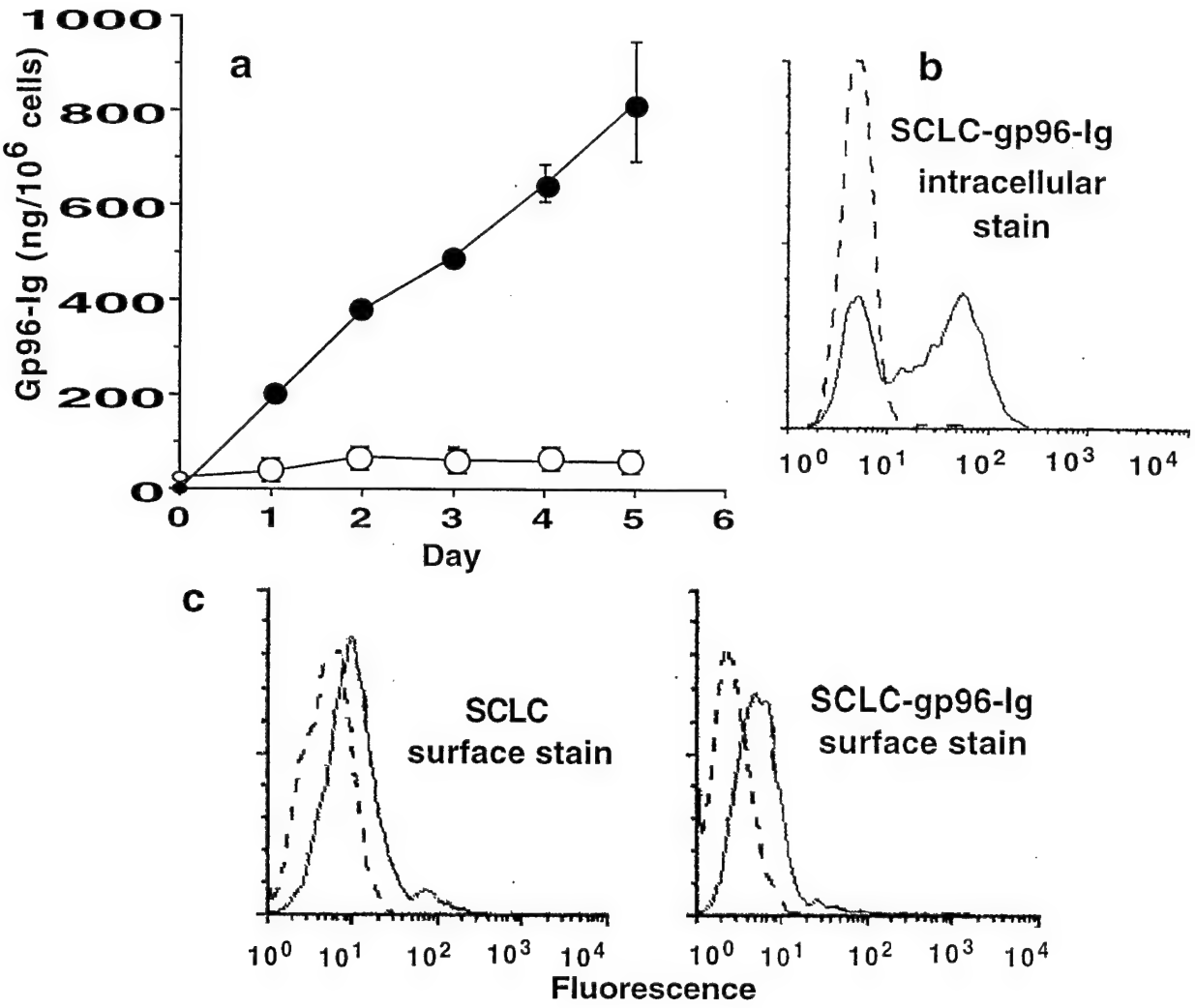


Figure 3, Yamazaki, et al.

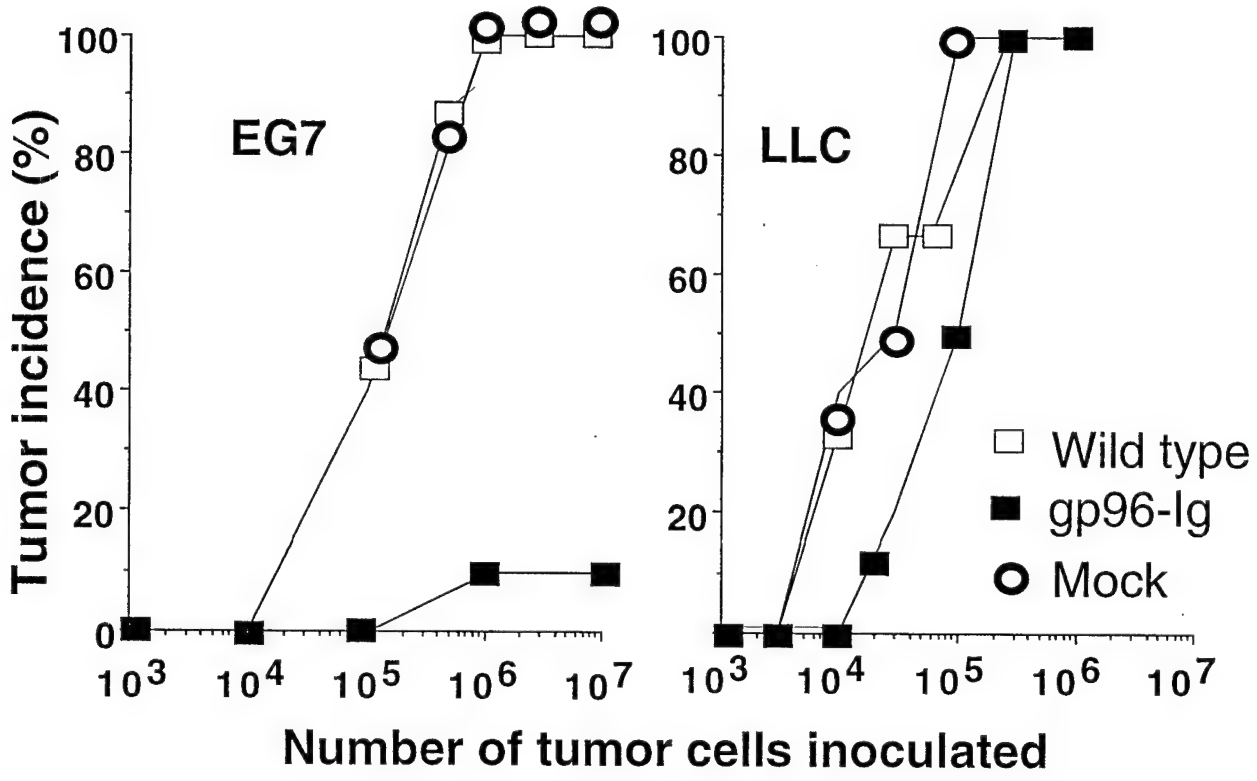


Figure 4, Yamazaki. et al.

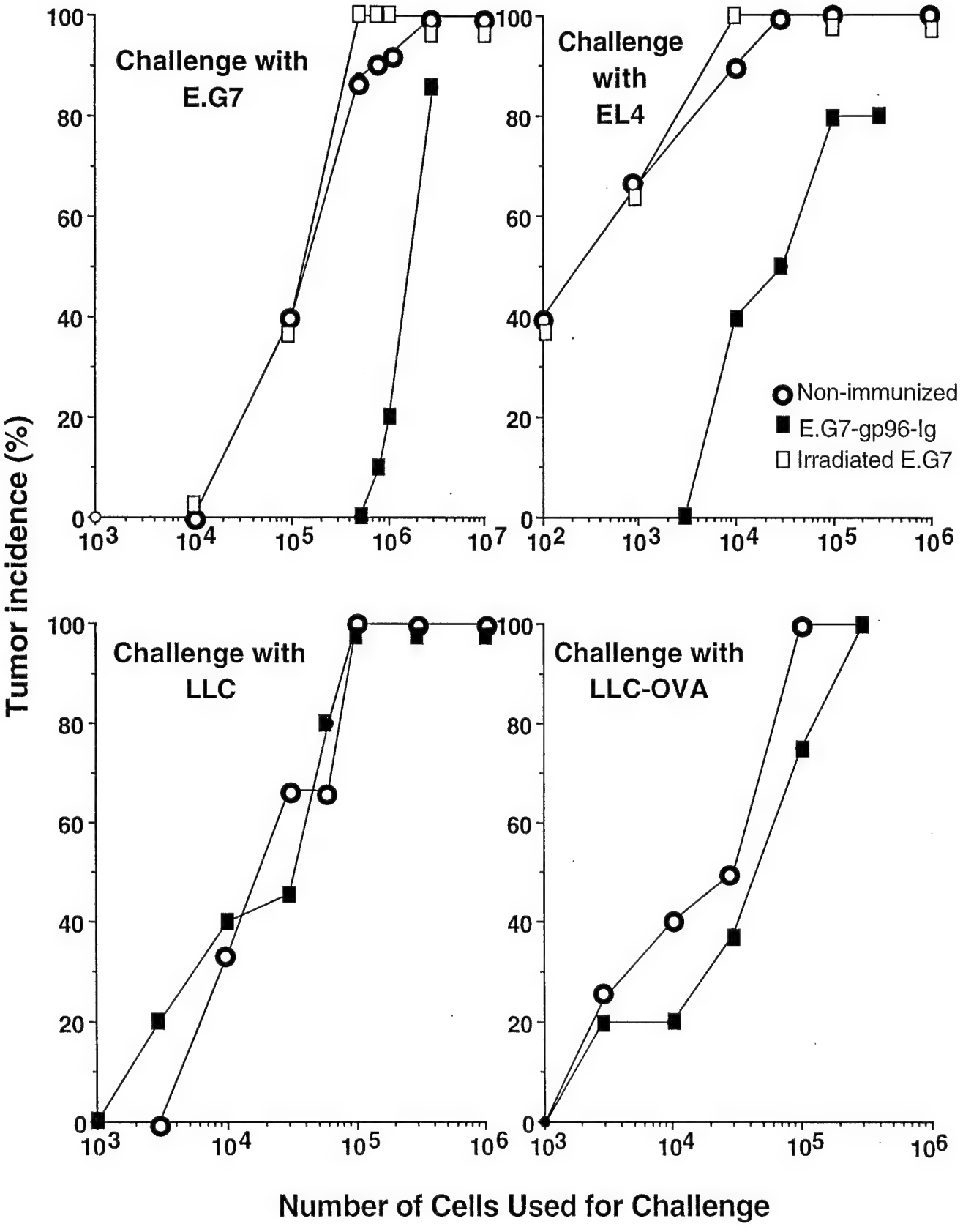
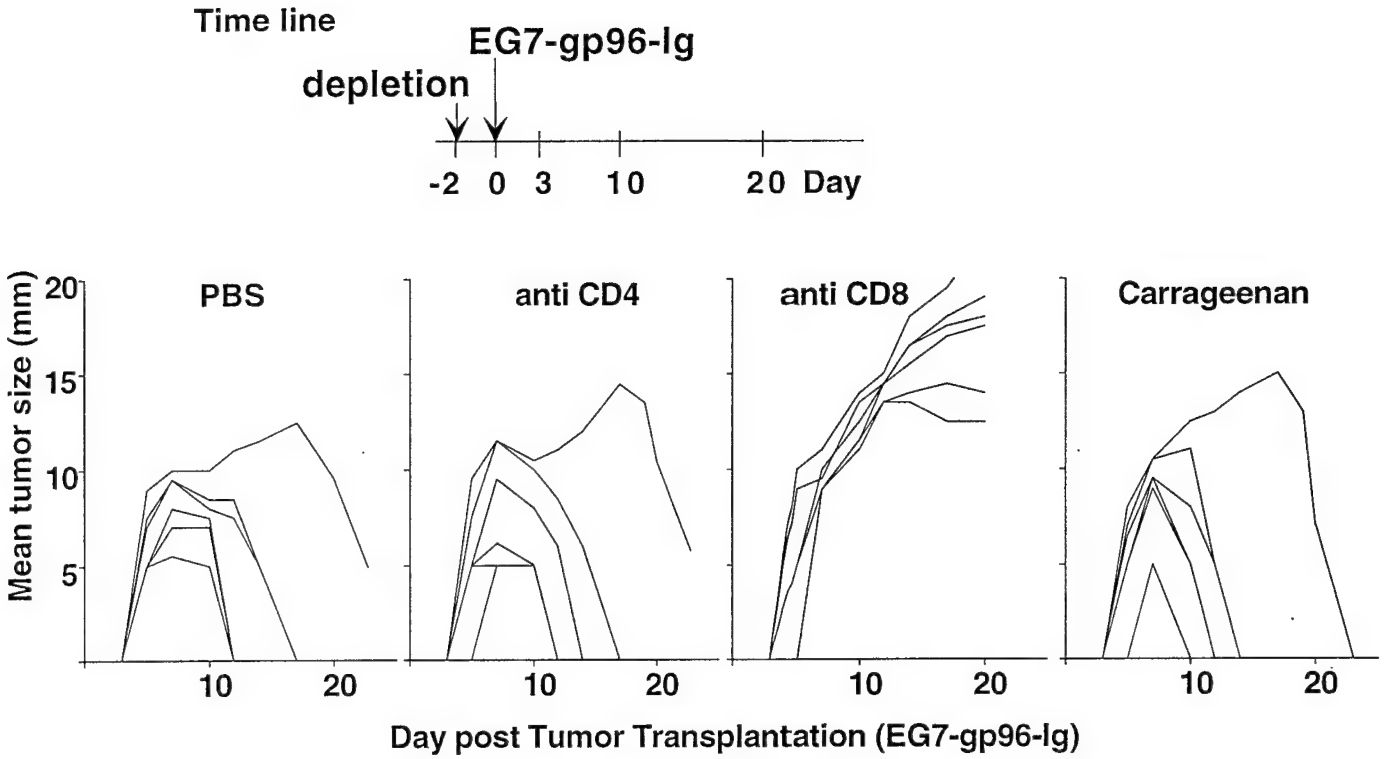


Figure 5, Yamazaki, et al.



Yamazaki et al; Figure 6

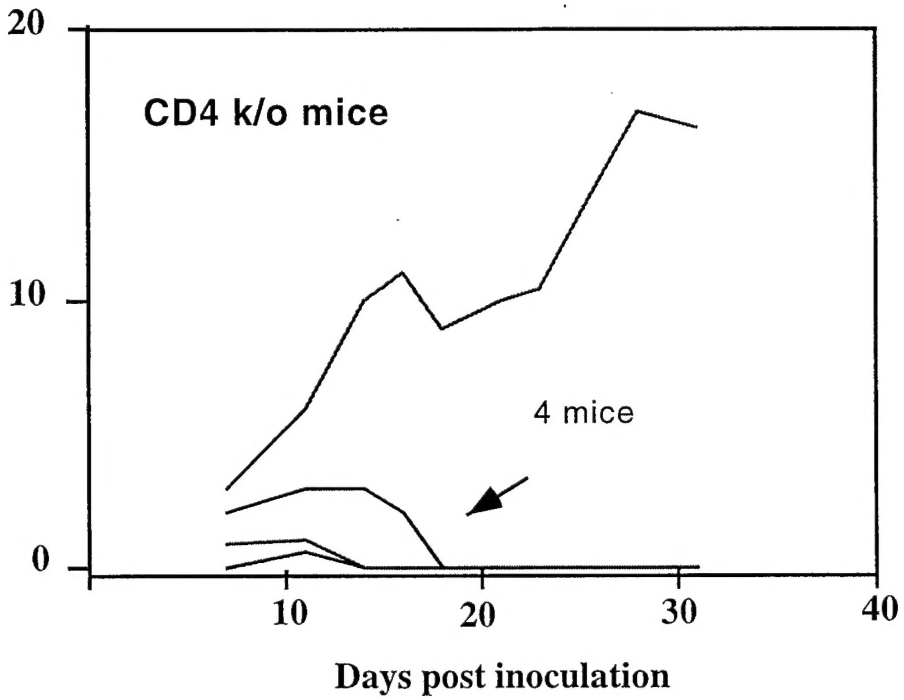
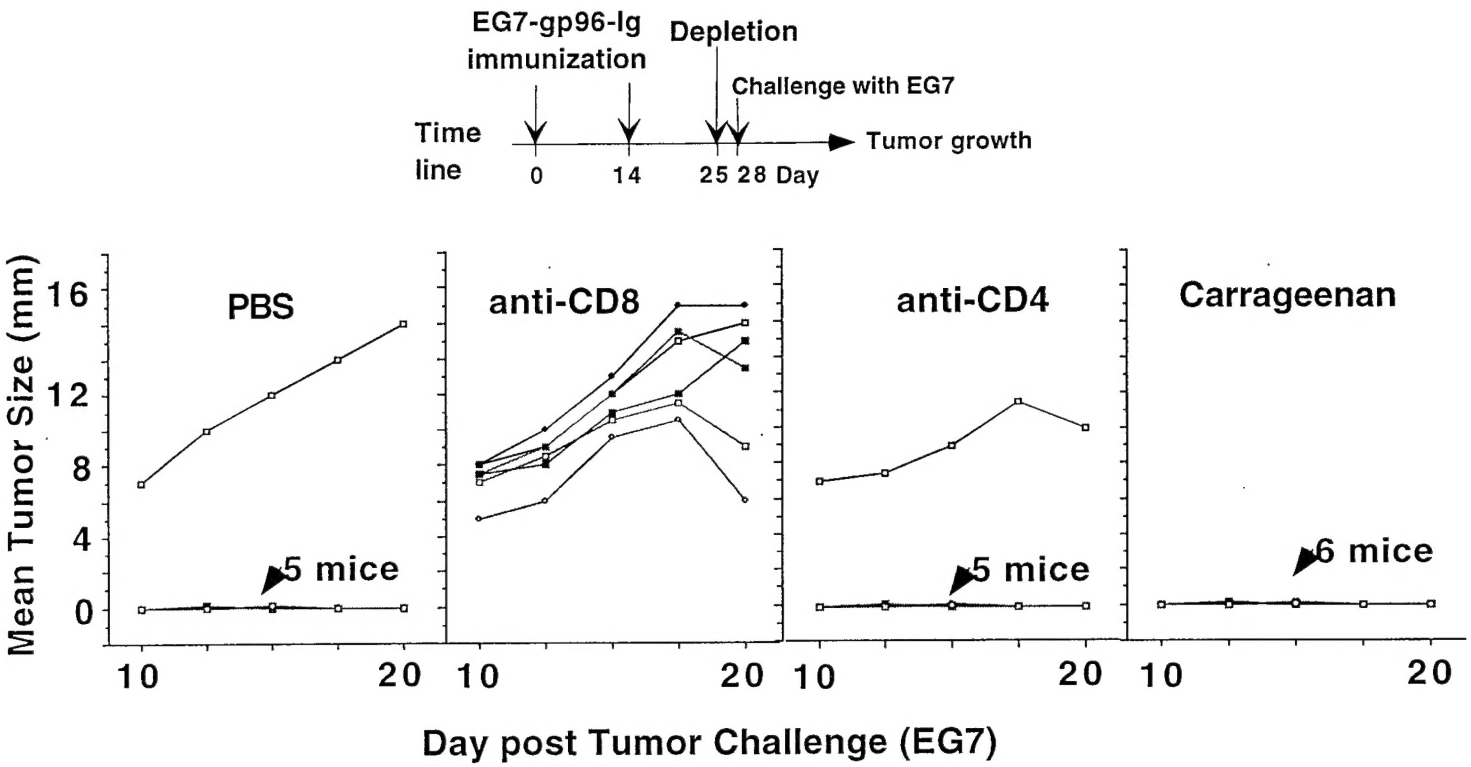


Fig. 7 Yamazaki et al



Medicine, Los Angeles 90095-1732

We have previously established a murine model of B c
sis, using premalignant cell lines (DACs) which are tumc
munodeficient mice, and malignant progressor daughter lin
tumorigenic in wildtype mice. RDA (Representational I
was employed to identify the differentially expressed gen
MVs. 853 genes were screened by multi-array expression a
sequencing, revealing 106 differentially expressed genes in four functional gene families. Surprisingly, most differences involved expression loss in the MV cells. One novel gene identified in this screen was GAS-2.24, which was highly expressed in DAC but not MV lines. GAS-2.24 is a new member of the GAS (growth-arrest-specific) gene family, the first associated with the lymphoid lineage. Members of this gene family encode a putative surface glycoprotein with four transmembrane domains. GAS-2.24 is expressed in normal B cell development, and is selectively lost in fully malignant B cell tumors and cell lines. GAS genes have been previously limited to the neuronal, epithelial, and mesenchymal cell lineages, and play roles in growth-regulatory signaling and intercellular junction formation. The normal developmental pattern of GAS-2.24 expression, and gene transfer of wild-type and dominant-negative GAS-2.24 on host-tumor interaction will be presented. Supported by NIH CA12800,the Lymphoma Research Foundation, and Jonsson Comprehensive Cancer Center.

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gp96 Engineered for Secretion of Tumor Peptides and for Vaccination against Cancer. K. Yamazaki, J. Spielman, G. Spruill, and E.R. Podack. Department of Microbiology and Immunology, University of Miami School of Medicine, Miami, Fl 33101

Mouse gp96 can induce specific immunity to the tumor from which it is isolated and may have great practical importance for vaccination and immunotherapy against cancer. In this study, we developed a generic vector expressing human gp96, by deleting the endoplasmic reticulum retention signal, KDEL, and adding the CH2 and CH3 domain of murine IgG1 in order to facilitate detection by ELISA and purification by affinity chromatography. After transfection, gp96-Ig was detected in the culture supernatant of NIH3T3, EL4, E.G7 (EL4 transfected with ovalbumin), LLC, P815, MC57, B16F10 and SCLC (small cell lung cancer) cell lines by ELISA. SDS PAGE of the purified product reveals 3-4 closely spaced 120kD bands. E.G7-gp96-Ig was rejected in C57BL/6 mice, while E.G7 developed tumor after subcutaneous injection. After two vaccinations of live E.G7-gp96-Ig, rechallenged E.G7 was rejected. However, gp96 purified from NIH3T3-gp96-Ig-Ova could not protect E.G7 rechallenge. These results suggest that gp96 secreted from E.G7-gp96-Ig holds tumor peptides of E.G7 and can induce tumor immunity against E.G7. These results also suggest that ovalbumin peptides are not as potent in inducing immunity as the mixed peptides produced by ovalbumin transfected EL4.

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IMMUNOLOGY/PRECLINICAL AND CLINICAL 5: Cancer Vaccines

#2055 Immunotherapy with an irradiated melanoma vaccine coupled with interleukin-12 significantly augments tumor control in a murine metastatic melanoma model. Shrayer, D.P., Cole, B., Hearing, V.J., Wolf, S.F., Wanebo, H.J. *Dept. of Surgery, Boston University and Dept. of Community Health, Brown University, Providence, RI 02908, Laboratory of Cell Biology, NCI, NIH, Bethesda, MD 20892 Genetics Institute, Inc., Cambridge MA 02139.*

Interleukin-12 can activate cytotoxic lymphocytes, stimulate natural killer cell activity, induce the production of INF-γ and inhibit the development of various experimental tumors. We have demonstrated that immunotherapy of melanoma bearing mice with an irradiated melanoma vaccine (IMV) coupled with IL-2 or GM-CSF had beneficial effects against primary melanoma growth and against subsequent spontaneous metastasis. We also have found that treatment of melanoma bearing mice with IL-12 (300 ng/day) for 4 weeks inhibited the development of primary melanoma tumors in 40% of mice. The purpose of this study was to investigate the efficacy of combined therapy of experimental melanoma with IMV prepared from B16F10 melanoma cells coupled with IL-12 treatment. C57BL/6 mice were challenged subcutaneously in the tail with B16F10 melanoma cells and by the 45th day, more that 50% of the mice had developed visible primary melanoma tumors at the injection site and received subsequent immunotherapy with IMV coupled with IL-12. Optimal effects on tumor growth parameters varied with IL-12 doses. Primary tumor growth was maximally inhibited in mice when IMV therapy was coupled with IL-12 at a dose of 50ng/day.* IMV combined with IL-12 at a dose of 100ng/day significantly reduced melanoma metastasis to the lungs.* Mean survival time was improved in mice treated with a combination of IMV with IL-12 at a dose of 300ng/day (87 days) vs. control (77 days).* *P-value = < 0.05

#2056 Cytotoxic peptide can elicit HLA-restricted CEA specific CTL from colorectal cancer patients *in vivo*–development of colorectal cancer vaccine. Tsunoda, T., Tanimura, H., Tanaka, H., Matsuda, K., Umano, Y., Shono, Y., Iwahashi, M., Yamaue, H., Takesako, K., Ideno, M. and Nukaya, I. *Second Dept. of Surgery, Wakayama Medical School, Wakayama 640-8156, and Takara Bio., Ohtsu, Shiga, Japan.*

To develop cancer vaccine, especially against colorectal cancer, CEA specific peptide bound by HLA-A*2402 has been used for immunization. We have already demonstrated that CEA peptide (CEA 652, TYACFVSNL, Binding HLA-A24 IC_{50} 5 nM) can elicit CTL for HLA-A*2402 positive, CEA-produced colon tumor cell lines from colon cancer patients *in vitro*. Clinical phase I trial was performed 5 eligible patients with colorectal cancer, which had high serum CEA level and HLA-A24 positive. From these patients, dendritic cell (DC) derived from peripheral blood mononuclear cells (PBMC) was used as a APC for CEA 652 peptide. Plastic-adhered PBMC cultured by IL-4 & GM-CSF from advanced colorectal cancer patients showed the activated DC from the view points of morphological and functional analysis. $1.6 - 9 \times 10^6$ of CEA 652 – pulsed DC were immunized for subcutaneous injection every week. Before and after vaccination, CTL activity and precursor frequency of PMBCs from 4 patients were assayed after simply 2 times stimulation. PBMC from 3 of 4 patients after vaccinations showed HLA-A24 restricted and CEA specific cytotoxic activity. Furthermore, by ELISPOT assay, precursor frequency increased after vaccination. Thus, it demonstrated that vaccination of CEA 652 – peptide pulsed DC was truly immunogenic and useful for clinical application.

#2057 Persistence of anti-transgene immunity in cancer patients following adenovirus-mediated transfer of the *lacZ* gene. Farace F., Molinier-Frenkel V., Le Boulaire C., Le Gal F.A., Gahéry-Segard H., Guillet J.G., Tursz T. *Institut Gustave-Roussy, Villejuif (FF, MFV, LC, TT) - U445, Hôpital Cochin, Paris (LFA, GSH, GJG) - France.*

We recently demonstrated that a single intratumor injection of 10^9pfu recombinant adenovirus (Ad-β-gal) in patients with inoperable lung cancer, induces strong short-term (1–3 months) humoral, helper (TH1 type) and cytotoxic T cell responses specific for the β-galactosidase protein. The objective of the present study was to evaluate the persistence of long-lasting anti-transgene immunity and in parallel, to assess patient immunocompetence revealed by responses to recall antigens (tetanus toxoid (TT), purified derivative protein (PDP)) or classic immunogens (EBV and influenza virus, allogeneic antigens in mixed lymphocytic reactions (MLR). β-gal-specific proliferative response declined rapidly (6 months) as did responses to the other antigens. In contrast, anti-β-gal humoral (IgG and IgA) responses persisted notably, up to 1 year (pre-mortem) as did responses to TT and poliomelytic antigens. While T cell effector cytotoxic responses specific for the viral peptides plummeted, the frequency of anti-β-gal CTL precursors remained particularly high, thus attesting major immunization. These results indicate that despite an overall loss of immunocompetence due to both advanced disease and chemotherapy, immune responses (mainly CTL) to the transgene protein vectorized by adenoviruses may be long lasting.

#2058 Induction of tumor immunity by gp96 secreted from engineered tumor cells. Yamazaki, K., Spielman, J., Spruill, G., Podack, E.R. *University of Miami, Miami, FL 33101.*

Gp96 is expected to have great practical importance on vaccination and immunotherapy against cancer patients of all MHC class I and II types. In this study, we developed a new construct of gp96, deleting the endoplasmic reticulum retention signal, KDEL, and replacing it with the Fc portion of murine IgG1 (gp96-Ig). KDEL deletion facilitated secretion of gp96-Ig from transfected cells and, after transfection, gp96-Ig was detected in the culture supernatant of E.G7 (EL4 transfected with ovalbumin) and LLC by ELISA using Ig tail as tag. SDS PAGE of the purified product reveals 3 closely spaced 120 kD bands. Cells transfected with and overexpressing gp96-Ig (E.G7-gp96-Ig and LLC-gp96-Ig) were less tumorigenic, compare to wild type tumors. Depletion studies showed that $CD8^+$ cells were required for E.G7-gp96-Ig rejection throughout induction phase and effector phase, but not $CD4^+$ cells nor macrophages. Immunization of mice with E.G7-gp96-Ig induced immunity to subsequent challenge with E.G7 and the tumors which have tumor antigens shared with E.G7 (EL4 and LLC-OVA), but not with an antigenically distinct tumor (LLC). These results suggest that gp96 secreted from E.G7-gp96-Ig holds peptides originated from antigens of E.G7 and can induce subsequent immunity. In addition, fusion of gp96 with murine IgG1 facilitated detection by FACS analysis by using Ig tail as tag, and purification by affinity chromatography on Protein A column. We now propose to generate gp96-Ig overexpressing cells by transfection with gp96-Ig cDNA for vaccination and immunotherapy against cancer.

#2059 Establishing an experimental murine model to study the mechanisms of E7-induced tumor that evades potent E7-specific immunotherapy. T-L Wang, H Ji, ZB Lu, S Xiang, RJ Kurman, DM Pardoll, and T-C Wu. *Departments of Pathology and Oncology, Johns Hopkins University, Baltimore, MD 21205.*

Current cancer immunotherapy is hampered by the development of immunoresistancy in tumor cells. Our previous immunotherapy studies demonstrated that vaccinia-Sig/E7/Lamp1 can effectively prevent and treat a human papillomavirus type 16 oncogene E7-expressing tumor (TC-1). In up to 20% of the mice, however, tumor develops. This study is to develop an experimental system that would enrich TC-1 tumor cells that can evade immunotherapy with vaccinia-Sig/E7/Lamp1. We challenged Sig/E7/Lamp1-vaccinated mice with TC-1 cells and selected the immunoresistant variant that developed into tumors. The tumors were isolated and then reinjected into the Sig/E7/Lamp1-vaccinated mice. Immunoresistant tumor cells were again selected. After 3 such cycles, the isolated TC-1 variant was designated P3. P3 cells developed into tumors in 100% of Sig/E7/Lamp1-vaccinated mice. Thus, we studied the mechanisms underlying the immunoresistance of P3 cells. RT-PCR and Western blot demonstrated that E7, the target of the immunotherapy, was still highly expressed in the P3 cells. ELISPOT and CTL assays by coculturing P3 or parental TC-1 with an E7-specific CTLs demonstrated that the recognition, and cytolytic activities of E7-specific CTLs toward P3 cells were significantly reduced as compared with the parental TC-1. Immunostaining of MHC class I revealed a slight decrease of MHC class I expression in P3 cells. However, although γ-IFN treatment can enhance the expression of MHC class I in P3, it still cannot restore the recognition of P3 by E7-specific CTLs. In summary, we established an E7-expressing TC-1 cell variant that can evade E7-specific immunotherapy. The cells lost the ability to activate the E7-specific CTLs. This system may serve as a model to identify cellular/genetic alterations in immunoresistant tumor cells developed during immunotherapy.

#2060 Isolation of HER-2/neu (HER2) specific T cell clones from a breast cancer patient following immunization with a HER2 peptide vaccine. Knutson, K.L., Crosby, P., and Disis, M.L. *Division of Oncology, University of Washington, Seattle, WA 98195.*

Isolation of tumor specific T cells from patients with cancer for characterization or expansion for potential treatments, such as adoptive immunotherapy, has been difficult. Tumor antigen vaccines may allow augmentation of the cancer specific T cell response, via immunization, to the point where isolation of tumor specific T cells for further characterization is feasible. T cell clones specific for HER2 HLA-A2 peptide p369–377 were isolated from a breast cancer patient who had been vaccinated with HER2 helper epitopes that contain HLA-A2-binding CTL epitopes within their sequences. Throughout the course of immunization, PBMCs from this patient showed strong proliferative responses to the HER2 helper epitope, p369–384. Furthermore, PBMCs from this patient lyse target cells loaded with HLA-A2 HER2 peptide p369–377; a 9-mer sequence encompassed in the longer helper epitope used to immunize the patient. Following vaccination, T cell clones specific for p369–377 were isolated by limiting dilution and characterized. A total of 21 p369–377 clones were generated from this patient. With the exception of two clones, all clones were CD3+. Eleven of the clones were CD8+/CD4−. Nine of the clones were CD4+/CD8−, despite being specific for an HLA-A2 binding peptide. The remaining 5 clones contained varying levels of both CD4 and CD8. The majority (19/21) of clones expressed the α/β T cell receptor, but interestingly, 2 clones expressed the γ/δ T cell receptor. Several of these clones could be induced to secrete interferon-γ (IFN-γ) in response to p369–377 peptide stimulation. While the majority of these IFN-γ-secreting clones were CD8+, one CD4 clone could secrete IFN-γ in response to the antigen. Several clones could lyse HLA-A2-transfected HER2-overexpressing tumor cells, including the γ/δ TCR expressing clones. The immunizing helper epitope, p369–384, is a sequence derived from the extracellular domain (ECD) of HER2. Limiting dilution